A High-Throughput System for Evaluating the Clinical Utility of Molecular Targets in Tissue Samples

1. Field of the Invention

The present invention is directed to a system and process for evaluating the potential clinical utility of molecular targets in tissue and, more particularly, to such a process that is fully automated so that large numbers of unique targets from multiple sources can be tested on large quantities of different tissue samples mounted on microscope slides.

BACKGROUND OF THE INVENTION

It is widely anticipated that twenty-first century medicine will become more individualized and that treatment strategies for each patient will be heavily influenced by their particular genotype or the genetic characteristics of the diseased tissue. This effort is often referred to as "personalized medicine" or "pharmacogenomics." Cancer, which arises from genetic abnormalities, is among the first diseases for which more individualized treatments are actively being sought. For any given therapeutic approach some cancer patients respond well while others fail to respond and may be seriously harmed by adverse side effects associated with the drug. Obtaining the genetic profile of a patient's tumor will play an increasingly important role in selecting the optimum course of therapy as many new drugs are being designed to act on specific molecular targets (DNA, RNA, or protein). Initially this information will be needed for selecting patients in clinical trials as it is projected that by 2005 one-half of all participants in clinical trials will be selected using pharmacogenomics based testing. (*Genetic Engineering News*, June 15, 1999 p.17). Ultimately most approved new pharmaceuticals are likely to have a corresponding molecular diagnostic test to be used in parallel with the drug.

One of the first of such drugs to enter the marketplace is HERCEPTIN® (Genentech, S. San Francisco, CA) a monoclonal antibody that targets metastatic breast cancer cells that overexpress the HER-2 oncogene. Approximately 25-30 percent of

women with breast cancer have cancers that overexpress the HER-2 oncogene, which is associated with more rapid cancer progression. HERCEPTIN® works by binding to the HER-2 growth factor receptors present in excessive amounts on the surface of the cancer cells. The drug is indicated only for patients whose turners have either amplification (i.e. extra copies) of the HER-2 gene as determined by *in-situ* by hybridization (ISH) or protein overexpression as determined by immunohistochemistry (IHC). HER-2 status has also been found to predict patient response to a variety of conventional therapeutic agents such as doxorubican.

Pharmaceutical and biotechnology companies are actively searching for new genetic markers that, like the HER-2 oncogene, can be used as both a target of therapy and as a tool for identifying patients most likely to benefit from drugs designed to interact with the target.

The combined efforts of participants in the Human Genome Project and the application of high throughput gene sequencing techniques has rapidly increased the number of genes available for research. Furthermore, new technologies have recently emerged to help identify or confirm the function of these genes and their utility as targets for therapeutic intervention. These technologies include serial analysis of gene expression (SAGE) and cDNA microarrays which measure the expression patterns of thousands of genes in one experiment and generate many potential targets for drug interaction.

While these new technologies can profile the expression patterns of hundreds of genes in parallel they can only survey one tissue sample at a time. However, analysis of hundreds of samples from patients in different stages of disease is needed to establish the potential clinical utility of a targeted gene or protein, especially genes believed to be involved in cancer since most oncogenes are amplified or overexpressed in multiple tumor types. Moreover, HER-2 and most other cancer markers are acquired (i.e. somatic) aberrations present only in the tumor. These markers are best identified in intact cells (*in-situ*) using ISH or IHC in order to allow the pathologist or scientist to study the histopathologic architecture or morphology of the tissue specimen under the microscope at the same time that the nucleic acid or proteins are being assayed. For example, ISH can be used to look for the presence of a genetic

abnormality or condition such as amplification of cancer causing genes specifically in cells that, when viewed under a microscope, morphologically appear to be malignant. If the target molecules sought to be detected are RNA sequences ISH permits researchers to learn where, and in what tissues, a particular gene is over or under expressed. Immunohistochemistry allows the detection of target antigens (usually proteins) within tissues. Antibodies to specific antigens are used to probe tissues to detect the target in specific cells of the tissue being assayed. Moreover, the level of protein expression and subcellular location of the target can also be elevated. Thus IHC can ascertain whether or not the targets are expressed in the cell nucleus, cytoplasm or on the cell membrane in a semi-quantitative manner.

In order to permit parallel *in-situ* detection of molecular targets in large numbers of tumor samples a "tissue microarray" was recently developed by U.S. government scientists as part of the Human Genome Project. (Kononen, J. et al. Tissue microarrays for high-throughput molecular profiling of tumor specimens. *Nature Med.* 7, 844-847 (1998)). As described elsewhere herein up to 1000 circular tissue biopsies from different tumors can be distributed in a grid format on a standard microscope slide. This allows nucleic acid probes or antibodies to be applied to hundreds of different samples at one time.

The advent of tissue microarrays notwithstanding, both ISH and IHC remain highly specialized techniques that are complex, time consuming, and vary from lab to lab. IHC requires a series of treatment steps conducted on a tissue section or cells (e.g. blood or bone marrow) mounted on a glass slide to highlight by selective staining certain morphological features indicative of disease states. Typical steps include pretreatment of the tissue section to remove the paraffin and reduce non-specific binding, retrieval of antigens masked by cross-linking of the proteins from the chemical fixatives, antibody treatment and incubation, enzyme labeled secondary antibody treatment and incubation, substrate reaction with the enzyme to produce a fluorophore or chromophore highlighting areas of the tissue section having epitopes binding with the antibody, counterstaining, and the like. Most of these steps are separated by multiple rinse steps to remove unreacted residual reagent from the prior step. Incubations can be conducted at elevated temperatures, usually around 3 7°C, and the

tissue must be continuously protected from dehydration. Lack of uniformity among different labs in their use of reagents and reaction conditions often yields to results that differ improperly.

ISH analysis, which relies upon the specific binding affinity of probes with unique or repetitive nucleotide sequences from the cells of tissue samples or infectious agents, requires a similar series of process steps with many different reagents and is further complicated by varying temperature requirements. As with antibodies each probe must be individually optimized for reactivity in tissue. The variables include probe length and labeling, probe concentration, hybridization conditions, stringency washes and detection methodology.

Most pharmaceutical and biotechnology companies that are identifying molecular targets are focusing their resources on developing new drugs and lack the motivation and infrastructure to conduct ISH and IHC in-house in a manner that is cost-effective and timely. Speed to market is one of the most critical factors in drug development for both biotechnology and pharmaceutical companies. It is estimated that more than $1 million in revenues can be lost for each day a major drug is delayed from reaching the market. (*Genetic Engineering News*, September 1, 1999). For life threatening diseases delays can also result in increased mortality and disease progression.

It is therefore apparent that there is a strong need for a system capable of performing high-throughput ISH and IHC on behalf of multiple pharmaceutical and biotechnology companies each having multiple unique molecular targets. There is also a need for a system capable of screening several hundred tissue samples with dozens of molecular targets to ascertain or validate the clinical utility of the targets in a timely and economic manner.

2. Description of the Related Art

SUMMARY OF THE INVENTION

The present invention is directed to apparatus and methods which can be employed in a high-throughput manner to establish the clinical utility of a molecular target by screening for the target in large quantities of tissues from different patients, organs, diseases, or disease stages. For each tissue sample the quantity or location of target is determined and compared to other samples from different organs or from patients in different disease states. For example, determining that amplification or overexpression of a particular gene is more frequent in tumors from patients with a recurrent form of cancer may create a prognostic marker used in planning treatment strategies as well as a target for designing new drugs that interact with the gene or its product.

The inventive apparatus and method are particularly adapted for providing services to various sources of potential target molecules, in particular pharmaceutical and biotechnology companies seeking ISH/IHC markers to help identify patients that would benefit from their drug as well as validation of their drug targets using *in-situ* techniques.

In its most preferred embodiment the apparatus according to the present invention generally comprises (i) a tissue microarray having hundreds of small tissue samples upon which reagents may be applied, (ii) an automated staining instrument for applying reagents to the tissue samples and automatically carrying out most of the steps required for ISH and IHC, and (iii) an imaging instrument to allow the user to readily ascertain the presence and/or quantity of target in each of the samples.

A key advantage of the present invention is the economies of scale and efficiencies created by performing essentially the same services using the same instrumentation for multiple clients having multiple targets to test or validate.

Another advantage of the present invention is the speed and high-throughput achieved through the use of the combination of tissue microarrays together with the automated staining instrumentation.

Still another advantage of the present invention is that it allows accurate comparison of results from multiple different tissue samples each having been treated in precisely the same manner.

Yet another advantage of the present invention is that each sample can receive an individualized staining or treatment protocol even when such protocols require different temperature parameters and other conditions.

Still another advantage of the present invention is that it allows the temperature of the entire surface of the slide to which the tissue is mounted to be carefully controlled (i.e. within plus or minus two degrees Celsius of the desired temperature). Such precision is particularly necessary for DNA denaturation and probe hybridization in ISH and related processes such as *in-situ* PCR. Furthermore, since the heating according to the present invention is made uniform, this narrow temperature range is maintained throughout the surface of the slide so that the tissue is evenly heated regardless of its position on the slide. This feature is particularly advantageous when heating tissue microarrays as it allows each of the plurality of tissue samples to be heated uniformly. This improves accuracy when comparing reactions in different tissue samples placed at opposite ends of the slide.

Still another advantage of the present invention is the negation of human error and increase in productivity resulting from automation.

Yet another advantage of the present invention is that the same staining protocol (reagents, times, temperatures, etc.) developed for evaluating or validating a target in a research setting can be subsequently employed a clinical (patient care) setting for disease prognosis or treatment selection.

With the foregoing and other objects, advantages and features of the invention that will become hereinafter apparent, the nature of the invention may be more clearly understood by reference to the following detailed description of the invention, the appended claims and to the several views illustrated in the drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a schematic illustration of the method according to the present invention.

FIGS. 2a-2c are perspective views of a tissue microarray according to the present invention and the instruments employed in creating a microarray from tissue blocks.

FIG. 3 is a perspective view of the staining instrument according to the present invention.

FIG. 4 is a perspective view of the staining instrument according to the present invention shown in conjunction with a computer and other instruments with which it operates.

FIG. 5 is a perspective of the staining instrument according to the present invention shown with a reagent dispenser.

FIG. 6 is an enlarged perspective view of a thermal platform according to the present invention shown supporting a tissue microarray slide.

DETAILED DESCRIPTION OF THE INVENTION

Referring now in detail to the drawings wherein like parts are designated by like reference numerals throughout, there is illustrated in FIG. 1 a schematic illustration of a preferred embodiment of the system and apparatus according to the present invention which is designated generally by reference numeral 10. System 10 generally comprises tissue microarray apparatus 12 for constructing arrays of hundreds of minute tissue samples mounted on a single glass microscope slide, staining apparatus 14 for automatically conducting most of the steps required for ISH/IHC, and imaging apparatus 16 to allow the results of the ISH/IHC staining to be visualized and analyzed by the user. System 10 preferably has access to one or more tissue banks 18 (a-c) having thousands of preserved surgical samples catalogued by organ type, disease, and patient history. In use and operation system 10 is adapted to serve multiple sources of different targets 20 such as pharmaceutical companies and the like who each supply the system with one or more molecular targets 22 (DNA, RNA, or protein) and receive data 24 regarding the clinical relevance of the targets based on screening of the tissue samples assayed.

Each of the aforementioned components of system 10 will now be described in more detail.

Definitions

The following terms shall have the following meanings as used herein:

"Automated" or "Automatic" means activity substantially computer controlled or machine driven and substantially free of human intervention during normal operation.

"Clinical Utility" means usefulness of a target for (i) designing or prescribing a drug or therapy that interacts with the target, or (ii) determining which patients would be most likely to benefit from a particular drug or therapy.

"Different Tissue" means tissue from different patients, organs, diseases, and/or disease stages.

"High-Throughput" means the capability to treat more than about 20,000 different tissue samples in one day.

"Sources" and "Target Sources" means companies or similar entities that provide the system according to the present invention with at least one target, receive services from the system, and are separately controlled from the company that uses the system.

"Target" and "Targeted molecules" means detectable molecules found in cells including without limitation nucleic acids, proteins, antigens, carbohydrates, lipids, and small molecules.

"Tissue" means any collection of cells that can be mounted on a standard glass microscope slide including, without limitation, sections of organs, tumor sections, bodily fluids, smears, frozen sections, cytology preps, and cell lines.

"Tissue Array" and "Tissue Microarray" means a glass microscope slide or similar solid surface having a plurality of different tissue samples thereupon.

"Screen" means determining the presence, absence, quantity, location, and/or other characteristics of a target in a tissue sample.

"Stain" means any biological or chemical substance which, when applied to targeted molecules in tissue, renders the molecules detectable under a microscope. Stains include without limitation detectable nucleic acid probes, antibodies, and dyes.

"Treating" or "Treatment" shall mean application of a stain to a tissue as well as other processes associated with such application including, without limitation, heating, cooling, washing, rinsing, drying, evaporation inhibition, deparaffinization, cell conditioning, mixing, incubating, and/or evaporation.

Tissue Microarray

In order to permit parallel *in-situ* detection of molecular targets in large numbers of tumor or other tissue samples a tissue microarray apparatus 12 is provided as illustrated in FIG. 2 (a-c). Developed at the National Human Genome Research Institute at the NIH (Bethesda, MD), apparatus 12 and the uses thereof to make a tissue microarray 15 having as many as 1000 cylindrical tissue biopsies distributed in a single array is described in Kononen, J. *et al.* "Tissue microarrays for high-throughput molecular profiling of tumor specimens." *Nature Med.* 7, 844-847 (1998) which is incorporated herein in its entirety.

Materials needed to construct the array (FIG. 2a) include a thin-walled stainless steel tube 25 sharpened like a cork borer for punching tissue core biopsies from donor block 26, stylet 27 for transferring core biopsies into recipient block 28 and adhesive tape 29. A hemotxylin & eosin (H&E) stained section 30 generated from donor block 26 is also provided to guide sampling from morphologically representative regions of the tissue.

A bar code label 17 is preferably applied to tissue microarray 15 for identification by the automated staining instrumentation 14. In lieu of a bar code other machine readable indicia or media may be employed including optically readable letters or symbols, magnetic strips, and the like.

Tissue Staining Instrumentation

In view of the large number of repetitive treatment steps needed for both IHC and ISH, automated staining instrumentation 14 is provided to significantly reduce human labor and the costs and error rate associated therewith, and to introduce uniformity. The preferred apparatus utilized is the DISCOVERY™ automated staining instrument available from Ventana Medical Systems, Inc. (Tucson, Arizona). This instrument is a microprocessor controlled system including a revolving carousel supporting radially positioned slides. A stepper motor rotates the carousel placing each slide under one of a series of reagent dispensers positioned above the slides. Bar codes on the slides and reagent dispensers permits the computer controlled positioning of the

dispensers and slides so that different reagent treatments can be performed for each of the various tissue samples by appropriate programming of the computer.

A unique feature of the DISCOVERY™ instrument that distinguishes it from other instruments known in the art, and renders it particularly suitable for inclusion in system 10 is that each slide rests on a separate heating platform allowing the temperature of each sample to be precisely controlled independent of the other samples. This feature is particularly advantageous when performing *in-situ* hybridization experiments which is nucleic acid based and requires higher and more precise temperature control. In order to denature the DNA double helix of both the target sample and the probe so as to render them single stranded, the temperature must be raised above the melting point of the duplex, usually about 65°- 105°C. At the same time it is imperative that the sample not be overheated past 100° C as doing so destroys cell morphology making it difficult to view under a microscope. Precise temperature control is also required in ISH to effect probe hybridization and washing at the desired stringency. The selected temperature must be low enough to enable hybridization between probe and template, but high enough to prevent mismatched hybrids from forming.

As shown in FIG. 4 instrumentation 14 comprises automated staining instrument 32, a host computer 33 preferably a dedicated personal computer, bulk fluid containers 34, waste container 36 and related equipment. A detailed description of instrumentation 14 is found in U.S. Patent Application Serial No. 09/259,240 to Richards et al. which corresponds to PCT International Application No. PCT/US99/04181, both filed on February 26, 1999 which are incorporated herein in their entirety. Instrument 32 is a computer controlled, bar code driven, staining instrument that automatically applies chemical and biological reagents to tissue or cells mounted or affixed to standard glass microscope slides or tissue microarrays. Up to 20 slides are mounted in a circular arrangement to a carousel which rotates, as directed by the computer, placing each slide under one of a series of reagent dispensers positioned above the slides. Each slide receives the selected reagents (e.g. DNA probe) and is washed, mixed and/or heated in an optimum sequence and for the required period of time. Tissue sections so stained or treated are then removed from the apparatus by the

user to be viewed under a microscope or other imaging instrument 16 by a scientist or pathologist who analyzes the stained sample to determine the presence, location, and quantity of target present. The computer controlled automation permits use of the apparatus in a "walk-away" manner, i.e. with little manual labor.

Individualized slide temperature control is accomplished by the heating system according to the present invention that has thermal platforms radially mounted to the carousel for heating the slides and sensing the temperature of each. A printed circuit board, also mounted to the slide carousel, individually monitors and controls each thermal platform separately. Information and power pass between the rotating carousel and the fixed apparatus via a slip ring assembly. This information includes the upper and lower temperature parameters needed for heating each of the 20 slides for the appropriate time period.

As best viewed in FIG. 3 instrument 32 comprises a housing 40 having a lid 42 hingedly mounted thereto. A slide carousel 44 is mounted within housing 40 for rotation in the direction of arrow A. As set forth in greater detail below, a plurality of thermal platforms 50 are mounted radially about the perimeter of carousel 44 upon which standard glass slides with tissue samples (standard or microarray) may be placed. It is a key feature of instrument 32 that the temperature of each slide may be individually controlled and is constant across the top surface of the slide. Also housed within instrument 32 are wash dispense nozzles, Liquid Coverslip™ dispense nozzle, fluid knife, wash volume adjust nozzle, bar code reader mirror, and air vortex mixers as shown and described in U.S. Patent Application Serial No 09/259,240 *supra*.

Rotatably mounted atop lid 42 is a reagent carousel 52. Dispensers 54 are removably mounted to a reagent tray 56 (FIG. 5) which, in turn, is adapted to engage carousel 52. Reagents may include any chemical or biological material conventionally applied to slides including nucleic acid probes or primers, polymerase, primary and secondary antibodies, digestion enzymes, pre-fixatives, post-fixatives, readout chemistry, and counterstains. However, for purposes of the present invention dispensers 54 are preferably modified so that the reagent is dispensed evenly over all of the minute tissue samples in microarray 15. This could be accomplished by adjusting

the orifice of the dispenser to widen the angle of spray or by moving the dispenser to drop reagent on two or more different locations of the microarray.

Reagent dispensers 54 are preferably bar code labeled 58 for identification by the computer. For each slide, a single reagent is applied and then incubated for a precise period of time in a temperature-controlled environment. Mixing of the reagents is accomplished by compressed air jets (not shown) aimed along the edge of the slide thus causing rotation of the reagent. After the appropriate incubation, the reagent is washed off the slide using nozzles. Then the remaining wash buffer volume is adjusted using the volume adjust nozzle. Liquid Coverslip™ solution, to inhibit evaporation, is then applied to the slide via nozzle. The air knife divides the pool of Liquid Coverslip™ followed by the application of the next reagent. These steps are repeated as the carousels turn until the protocol is completed.

Instrument 14 permits the automatic staining or treating of multiple tissue samples mounted on microscope slides so that each sample can receive an individualized staining or treatment protocol even when such protocols require different temperature parameters. Thus, different DNA probe and/or antibody based staining procedures can be run simultaneously despite the fact that each may have different heating requirements at a given point in time. Additionally, samples requiring de-waxing (e.g. tumor sections) can be automatically processed at the same time as other samples (e.g. smears) that do not require this preliminary step.

A difficulty frequently encountered in both IHC and ISH testing results from the manner in which the tissues are typically preserved. The mainstay of the diagnostic pathology laboratory has been for many decades the formalin-fixed, paraffin embedded block of tissue, sectioned and mounted upon glass slides. Fixation in such a preservative causes cross-linking of macromolecules found in both peptides and DNA. These cross-linked components must be removed to allow access of the probe to the target nucleic acid and to allow the antibody to recognize the corresponding antigen. "Unmasking" the antigen and/or nucleic acid is typically accomplished manually with multiple pretreatment, protolytic digestion, and wash steps. It is part of the preferred embodiment of the present invention to include the process of conditioning cells so that their antigens and nucleic acids are available for detection could be automated.

Prior to staining, complete removal of the paraffin, a hydrophobic substance, is also required so that it does not interfere with antibody or probe binding. Deparaffinization is normally achieved by the use of two or three successive clearing reagents that are paraffin solvents such as xylene, xylene substitutes or toluene which may be toxic, flammable and pose environmental hazards. Safer and faster methods to deparaffinize the slides would be advantageous and may be used.

In order to treat the plurality of tissue mounted to microarray some of the treatment parameters may have to be changed from those employed when treating conventional tissue section. These changes may include, for example, more time for cell conditioning to restore the three dimensional structure of the tissue. Additionally the reagent concentrations may need to be increased when staining microarrays due to the quantity of target that is present from that typically present with conventional sections.

Turning now to FIG. 6, about twenty thermal platforms 50 are radially mounted to carousel 44 for heating the slides and monitoring the temperature thereof. Each thermal platform 50 comprise a plate 52 made of brass or a similar material that conducts heat upon which a glass microscope slide may rest. A control electronics printed circuit board (not shown) is also mounted to the slide carousel for monitoring the sensors and controlling the heaters. Information and power are transferred from the fixed instrument platform to the rotating carousel via a slip ring assembly. This information includes the temperature parameters needed for heating the slides (upper and lower) communicated from a microprocessor (after having been downloaded from computer 32) to control electronics as described in the aforementioned application to Richards et al. If, during a run, the slide temperature is determined to be below the programmed lower limit, the thermal platform heats the slide. Likewise, if the slide temperature is found to be above the upper limit, heating is stopped. A power supply of sufficient capacity to provide about eight watts per heater is provided to meet the requisite rate of temperature rise (a/k/a "ramp up").

Heating the slide surface uniformly is a key goal of the present invention since tissue specimens in a microarray are mounted at different positions on the slide. This poses a challenge for conduction heating, even when done manually, since traditional

hot plates often generate patchy "hotspots" making it hard to know where to place the slide on the plate. If cells in different tissue samples are not heating evenly the slide cannot be reliably interpreted. For example if the temperature on one side of the slide is not high enough to denature the probe or tissue DNA this may lead to false negatives for samples positions at that side.

In order to ensure uniform heating by thermal platform 50, the resistive heater (not shown) is bonded to the bottom side of plate 52. The brass plate has sufficient conductivity to smooth out any local non-uniformity caused by the fact that the heating traces are not continuous over the surface, but rather are adjacent lines separated by a space where no heat is generated. The separation in on the order of .015 inch, so this non-uniformity in the heat source is not felt at the side of the plate which abuts the slide.

Uniformity of temperature of the slide should also be achieved by modifying the heating traces so that they produce heat in a non-uniform manner. More heat is dissipated to the surrounding air at the edges of the plate than in the center. To compensate for this phenomenon the heater flux in the center of the slide should be adjusted in the manner described in the application to Richards, et al. *infra* at page 14. As a result the temperature differential between samples positioned anywhere on the slide should be within plus or minus two degrees Celsius.

In sum, staining instrument 14 is substantially the same as the instrument described in USSN 09/259,240 except for the aforementioned modifications to dispenser 54 and the detection chemistries in order to be used in conjunction with tissue microarray 15.

Other Components

Imaging equipment 16 for viewing and interpreting the assays performed on the tissue array section are preferably provided as part of system 10. Equipment 16 may comprise a standard microscope (fluorescent or brightfield) or a digital imaging system such as those employed in imaging DNA arrays. Such a digital imaging system would likely comprise an electronic camera, a frame grabber, and image processing software installed on a personal computer (PC). A variety of low-cost electronic cameras based

on charge-coupled device (CCD) technology are commercially available. A frame grabber is a printed circuit board that can be added to a PC as an interface between the camera and the software. The image processing software uses picture data stored in the frame grabber and is commercially available. Automated readers and optical scanners may also be employed to assist in interpreting the stained slide.

System 10 preferably has access to one or more tissue banks 18 (a-c) consisting of standard blocks of paraffin embedded tissue as has been utilized for decades. The blocks should be readily accessible by disease type and stage, patient history at the time the tissue was excised, and preferably the post-surgical clinical follow-up. It is also desirable that the blocks identify the method of fixation (e.g. formalin) as well as the results any diagnostic tests performed. Such banks exists at most hospitals having a surgical pathology department. For example, an extensive 100 year old tissue bank exists at the Johns Hopkins University Medical Center (Baltimore, Maryland).

Use and Operation

Sources 20 of target molecules for system 10 would include pharmaceutical and biotechnology companies, research institutes, and university laboratories that have identified novel targets believed to be associated with a particular disease or disorder including genes, gene fragments, mRNA sequences, or antigens. Typically they have an idea or prediction of the targets' biological function from profiling the expression pattern of clinical samples using one or more of the aforementioned technologies such as SAGE or DNA microarrays.

With this data the user of system 10 would access tissue banks 18 and select between 30 and 1000 blocks representing different patient populations and disease states. The selected blocks are used as donor blocks 26. The types of tissue samples selected would depend largely on the diseases for which new *in situ* assays would be deemed useful in medical practice. This would include cancer, arthritis, and skin disorders such as psoriasis and eczema. This might also include tissues from patients diagnosed Chron's disease, type I diabetes, and certain other autoimmune disorders. Additionally, tissue infected with a pathogen (e.g. Human Papaloma Virus) would be

used for developing *in situ* assays for selecting treatments to various infectious diseases.

An example of the construction of a microarrays, including the selection of appropriate tissue samples for screening tumors, is disclosed by Schraml et al, *Clinical Cancer Research*, Vol. 5 1966-1975, August 1999 which is incorporated herein in its entirety.

A H&E stained section 30 [FIG. 2(a)] is made from each block to define representative tumor regions. Tissue cylinders with a diameter 0.6mm are punched from tumor areas of each "donor" tissue block and brought into a recipient paraffin block using steel tube 25. Five-μm sections of the resulting multitumor tissue microarray block are transferred to glass slides using the paraffin sectioning aid system [adhesive coated slides (PSA-CS4x), adhesive tape, and UV lamp; Intermedics, Inc. Hackensack, NJ] supporting the cohesion of 0.6 mm array elements.

If the target is either a DNA or RNA sequence, a nucleic acid probe is constructed that will preferentially hybridize with the target. The techniques of probe construction are described by Herrington and McGee, *Diagnostic Molecular Pathology*, IRL Press (1992).

If the target is a protein, a detectable antibody is raised that will preferentially bind to the target. The techniques of raising antibodies are described in Lidell and Cryer, *A Practical Guide to Monoclonal Antibodies* (1991).

Sections cut from the array allow parallel detection of DNA (fluorescense *in situ* hybridization, FISH), RNA (mRNA ISH) or protein (immunohistochemistry, IHC) targets in each of the hundreds of specimens in the array. Preferably staining instrument 14 is employed to carry out the staining protocols in an automated manner. Alternatively, manual staining of the microarray may first be employed followed by automatic staining of conventional samples with instrumentation 14 to confirm the results of the array.

Staining instrument 14 may be used to perform *in-situ* hybridization (ISH), *in-situ*, PCR, immunohistochemistry (IHC), Special Stains, as well as a variety of chemical (non-biological) tissue staining techniques on an array or conventional tissue specimens. Moreover, two or more of the above techniques may be employed during a

single run despite their differing temperature requirements due to the inventive heating system herein.

In-situ hybridization is clearly a technique that may be advantageously employed with the instrument according to the present invention, either alone or in combination with other techniques, since many of the steps in ISH must be carefully temperature controlled for a precise period of time. The precise amount of heat for a specific period of time is necessary to sufficiently denature the DNA so that subsequent hybridization may occur without over-heating to the point where cell morphology is degraded. Different specimens may require different times and temperatures for denaturation depending on the tissue type and how it was prepared and fixed. The steps of denaturation, hybridization, and posthybridization washes each have unique temperature and time requirements that depend on the particulars of the probe and tissue being tested. DNA probes require and are typically hybridized at between 30°-65°C while RNA probes are typically hybridized at higher temperatures with the time for hybridization varying from 30 min. to 24 hours depending on target copy number, probe size and specimen type. Standard denaturation for cytogenetic preparations is performed at about 72°C for 2 min. while for tissue sections the conditions may vary from 55°C to 95°C from 2 to 30 min. Post hybridization wash temperatures may vary from about 65°C to 100°C for 2 min. to 15 min. Salt concentration may vary from 0.1x to 2x SSC. Probe detection temperatures may vary from ambient to 42°C for 2 min. to 30 min.

The low mass of the plate 60 and heater 64 enables the rapid heating and cooling of the slide and consequently the tissue on the slide (i.e. from 37°C to 95°C in 180 seconds). The increased rapidity of heating and cooling increases the efficiency of *in situ* hybridization. Concomitantly the background is decreased and the quality of the resulting test is vastly improved.

Hybridization or denaturation of DNA is absolutely essential to an ISH tissue staining process and requires that temperatures in the range of 92-100 degrees C be quickly reached, precisely controlled and maintained. The thermal platform brings treated tissue on microscope slides to the required temperature range in less than 180 seconds with an accuracy of plus or minus 2 degrees C. Rapid loss of temperature in

hybridized tissue is essential to successful staining and diagnosis. A fan or other rapid cooling feature may be added to bring the required temperature to 37 degrees C in less than 420 seconds.

The apparatus permits the placement of multiple types of specimens and ISH tests in the same run without compromising the unique requirements of each ISH test requirement (i.e., hybridization temperature 37-45°C stringency, and wash concentrations). The system may run more than one detection chemistry in the same run on different slides. As used herein "ISH" includes both fluorescent detection (FISH) and non-fluorescent detection (e.g. brightfield detection).

Apparatus 10 may also be employed for the simultaneous application of ISH and IHC staining to certain tissue sections to allow both genetic and protein abnormalities to be viewed at the same time. This may be advantageous, for example, in assaying breast tumor sections for both gene amplification and protein expression of HER-2/neu as both have been deemed to have clinical significance. See Ross et al. "The Her-2/neu Oncogene in Breast Cancer," *The Oncologist* 1998; 3:237-253.

Sections embedded in paraffin require as an early step deparaffinization of the embedded tissue. Using the thermal platform eliminates the use of harsh chemicals such as xylene, through the use of precisely controlled heating of individual slides allowing the paraffin embedded in the tissue to melt out and float in aqueous solution where it can be rinsed away. Paraffin, being less dense than water, once liquified rises through the aqueous buffer on the tissue sample and floats on top of this fluid. The liquid paraffin can then be removed from the microscope slide and away from the tissue sample by passing a fluid stream, either liquid or gaseous, over the liquid paraffin. Details of this procedure are set forth in U.S. Patent Application Serial No. 60/099,018 filed September 3, 1998 which is incorporated herein. A similar technique may be employed to remove embedding materials other than paraffin such as plastics although the addition of etching reagents may be required.

Heating the tissue with thermal platforms 50 in an appropriate aqueous solution has been found to improve the accessibility of the stain to the target molecule in the cell (protein, nucleic acid, carbohydrate, lipid, or small molecule). Lack of accessibility may be caused by cross-linking of the molecules by aldehydes used in preserving the

tissue or by other changes in the confirmation caused by fixatives. Cross-linking of antigens causes a loss of antigenicity due to the chemical modification of antigenic proteins. This process of improving accessibility of the stain (biological or chemical) to the molecular target is referred to herein as "cell conditioning." For protein targets the preferred conditioning solution is citrate buffer, the preferred temperature is up to about 95 degrees C, and the preferred time of heating is about one hour. For protein targets the preferred conditioning solution is citrate buffer and the preferred temperature is up to about 100 degrees C for about 42 minutes. Heating the tissue sample by the thermal platform decreases the degree of cross-linking in aldehyde treated tissue such that the modified antigen reverts to a form recognizable by a corresponding antibody thereby enhancing the staining. For RNA targets the preferred conditioning solution is citrate buffer and the preferred temperature is up to about 75 degrees C for about one hour. Many alternatives to citrate buffer may be employed as cell conditioning solution. A list of such solutions appears in *Analytical Morphology*, Gu, ed., Eaton Publishing Co. (1997) at pp. 1-40. The solutions should generally have known molarity, pH, and composition. Sodium dodecyl sulfate (SDS), ethylene glycol are preferably added to the conditioning solution.

Typical In-Situ Hybridization (ISH), In-Situ PCR, Immunohistochemical (IHC), Histochemical (HC), or Enzymehistochemical (EHC) methods as carried out with the apparatus of this invention includes the following steps are described in the patent application of Richards et al. *infra*.

The stained slides would be scored and analyzed by a pathologist or pathology support personnel using techniques known in the art. The results would be preferably be correlated by a biostatistician to arrive at clinical utility of the target in tissue. For example, it might be determined that overexpression of the gene target is a particular tumor type correlates with extended survival in patients treated with a drug designed to block expression of the gene target. A useful *in situ* assay could then be developed for use in selecting patients to receive the drug.

System 10 should be capable of screening large volumes of tissue samples in a high-throughput manner. If both tissue microarray 12 and automated staining instrumentation 14 are used at least one run and perhaps two runs of twenty slides,

each supporting up to 1000 minute tissue samples may be treated in one day. Thus between 20,000 and 40,000 different samples may be screened per day using system 10.

Although certain presently preferred embodiments of the invention have been described herein, it will be apparent to those skilled in the art to which the invention pertains that variations and modifications of the described embodiment may be made without departing from the spirit and scope of the invention. Accordingly, it is intended that the invention be limited only to the extent required by the appended claims and the applicable rules of law. The references cited above are hereby incorporated herein in their entirety.